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PROTEINS HAVING IMPROVED ORAL BIOAVAILABILITY

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PROTEINS HAVING IMPROVED ORAL BIOAVAILABILITY

FIELD OF THE INVENTION

The present invention relates to the administration by the oral route of RANTES, MIP-1alpha, MIP-1beta as well as their muteins having at least 90% homology with the corresponding wild-type (WT) molecule for treating or preventing autoimmune and inflammatory diseases as well as bacterial and viral infections.

BACKGROUND OF THE INVENTION

Chemokines constitute a family of small pro-inflammatory cytokines with leukocyte chemotactic and activating properties. Depending on the position of the first conserved cysteines, the chemokine family can be divided in C-C, C-X-C and C-X_3-C chemokines (Baggiolini M. et al., Adv Immunol. 1994, 55:97-179; Baggiolini M. et al., Annu Rev Immunol. 1997,15:675-705; Taub D. et al., Cytokine Growth Factor Rev. 1996,7(4):355-76).

Many C-X-C chemokines such as interleukin-8 (IL-8) are chemotactic for neutrophils, while C-C chemokines are active on a variety of leukocytes including monocytes, lymphocytes, eosinophils, basophils, NK cells and dendritic cells.

The NH_2-terminal domain of chemokines is involved in receptor binding and NH_2-terminal processing can either activate chemokines or render chemokines completely inactive.

N-terminal variants of synthetical C-C chemokines have been tested for their activity as inhibitors or antagonists of the naturally occurring forms. MCP-1, MCP-3 and RANTES missing the 8 to 9 NH_2-terminal amino acids are inactive on monocytes and are useful as receptor antagonists (Gong JH et al., J Exp Med. 1995,181(2):631-40 and Gong JH et al., J Biol Chem. 1996, 271(18):10521-7).

Extension of RANTES with one methionine results in almost complete inactivation of the molecule and Met-RANTES behaves as an antagonist for the authentic one (Proudfoot AE et al., J Biol Chem. 1996 Feb 2;271(5):2599-603).

WO 99/16877 relates to amino-terminally truncated RANTES, lacking NH_2-terminal amino acids corresponding to amino acid residues 1, 1-2, 1-3 or 1-4 of the naturally-occurring RANTES and having chemokine antagonistic activity, as well as cDNA sequences encoding them, their use in therapy and/or in diagnosis of the

diseases, in which an antagonistic activity of the chemokine effects is required. RANTES (3-68) is the preferred truncated chemokine antagonist.

Even if the chemoattractant activity of RANTES and of CC chemokines in general has been studied mainly in connection with the specific cell membrane receptors, RANTES can interact also with Glycosaminoglycans (GAGs), highly variable, branched sugar groups added post-translationally to several proteins, generically called proteoglycans (PGs). Such proteins are present on cell membrane, in the extracellular matrix and in the blood steam, where isolated GAGs can also be present.

The interaction with GAGs is a feature common to many cell-signaling soluble molecules (interleukins, growth factors). PGs, or isolated GAGs, can form a complex with soluble molecules, probably at the scope to protect this molecule from proteolysis in the extracellular environment. It has been also proposed that GAGs may help the correct presentation of cell signaling molecules to their specific receptor and, eventually, also the modulation of target cell activation.

In the case of chemokines, the concentration into immobilized gradients at the site of inflammation and, consequently, the interaction with cell receptors and their activation state seem to be modulated by the different forms of GAGs (Hoogewerf AJ et al., Biochemistry 1997, 36(44):13570-8). Therefore, it has been suggested that the modulation of the such interactions may represent a therapeutic approach in inflammatory disease (Schwarz MK and Wells TN, Curr Opin Chem Biol. 1999, 3(4):407-17) and in HIV infection (Burns JM et al., Proc Natl Acad Sci U S A 1999, 96(25):14499-504).

The structural requirements and functional effects of GAG-RANTES interaction have been studied in various models. RANTES binds GAGs on human umbilical vein endothelial cells (HUVECs) at micromolar concentrations with an affinity and a specificity higher then other chemokines, like MCP-1, IL-8, or MIP-1alpha. Such interaction appears to be not simply electrostatic but also depending by other parameters like length and N- and O-sulfation of the GAGs (Kuschert GS et al., Biochemistry 1999, 38(39):12959-68). GAG-defective cell lines still can bind chemokines but the presence of cell surface GAGs greatly enhances their activity on the receptors when they are at low concentrations (Ali S et al., J Biol Chem 2000, 275(16):11721-7). Other experiments showed that GAGs, heparin sulphate in particular, facilitate the interaction of RANTES with the cell surface of macrophages

and the consequent inhibition of HIV infection, a result consistent with the well-known resistance of these cells, poorly expressing heparin sulphate, to antiviral effects of RANTES (Oravecz T, et al., J Immunol. 1997,159(9):4587-92).

Soluble GAGs compete with cell membrane GAGs, and they can act as specific inhibitors of RANTES-induced activation surface (Appay V, et al.; Int Immunol 2000, 12(8):1173-82), or as suppressor HIV infection (Burns JM, et al.; Proc Natl Acad Sci U S A 1999, 96(25):14499-504).

RANTES contains a cationic site (RKNR) at residues 44-47 which is conserved in the GAG binding domain of other chemokines, like MIP-1alpha (Koopmann W and Krangel MS, J. Biol. Chem. 1997, 272(15):10103-9) and MIP-1beta (Koopmann W et al., J Immunol. 1999, 163(4):2120-7).

Human RANTES variants containing single mutations in these cationic sites have been disclosed as RANTES antagonists having potential therapeutic applications in the treatment of HIV infection and inflammatory or allergic diseases (WO 99/33989).

It has also been disclosed that a triple mutant of RANTES, in which three residues at positions 44, 45 and 47 have been substituted with Alanine, has lost the GAG-binding ability and it is useful in the treatment of multiple sclerosis (MS) and/or other demyelinating diseases (see PCT/EP 01/11428).

DESCRIPTION OF THE INVENTION

All the peptides and proteins known until now, including those which have become commercialized drug products, lack oral efficacy and therefore have always been administered by parenteral route. Injections are generally performed by the physician or by the medical professional staff and the patients are expected to visit a surgery or a hospital regularly in order to receive treatment. Besides the discomfort created, the time taken up by this type of application often leads to unsatisfactory compliance by the patient, particularly when the treatment extends over several months.

It has now been found that some CC chemokines can be efficaciously administered by oral route, thus rendering possible the self-administration by the patient and consequently improving patients' cooperation and compliance. These advantages are even more evident in the case of a long-term therapy, such as the ones of chronic inflammatory and autoimmune diseases. Another advantage of the oral administration lies

in a substantially lower complications' rate due to possible side effects, such as local inflammation, abscess formation and nerve lesions, which can be observed in cases of injections. Additional advantages will become evident from the description, which follows.

The CC chemokines that have been shown to be orally bioavailable are of RANTES, MIP-1alpha, MIP-1beta as well as their muteins having at least 90% homology with the corresponding wild-type (WT) molecule, preferably having from 95 to 99% of homology.

Some examples of these muteins include all the muteins, which comprise at least 2 mutations with respect to WT molecule in their GAG-binding domain and that, by way of this mutation have a reduced GAG-binding activity. Indeed these muteins are the preferred ones according to the invention.

The wording "a reduced GAG-binding activity" means that the mutants of the invention have a lower ability to bind to GAGs, i.e. a lower percentage of each of these mutants bind to GAGs (like heparin sulphate) with respect to the corresponding wild-type molecule.

More preferably are mutants of human RANTES, in which the three basic amino acids at positions 44, 45 and 47 of the wild-type molecule have been substituted by other amino acids. Such residues can be substituted with small, aliphatic, non-polar or slightly polar residues, such as for example Ala, Ser, Thr, Pro, and Gly. Alanine is the preferred one.

Some examples of these muteins having a reduced GAG-binding activity are those comprising an amino acid sequence selected from the group consisting of SEQ ID 1, 2, 3, 4, 5 and 6. Other muteins included in the above definition are those muteins that contain 1 or 2 amino acid deletion or extension at the N-terminus. Some example of this last kind of muteins are those comprising an amino acid sequence selected from the group consisting of SEQ ID: 3, 7 and 11.

Therefore the main object of the present invention is the use of the RANTES, MIP-1alpha, MIP-1beta as well as their muteins having at least 90% homology with the corresponding wild-type (WT) molecule to produce a pharmaceutical composition for treating or preventing autoimmune and inflammatory diseases as well as bacterial and viral infections by oral administration. Non-limitative examples of such diseases are the following: arthritis, rheumatoid arthritis (RA), psoriatic arthritis, osteoarthritis, systemic lupus erythematosus (SLE), systemic sclerosis, scleroderma, polymyositis, glomerulonephritis, fibrosis, lung fibrosis, allergic or hypersensitvity diseases,

dermatitis, Type IV hypersensitivity also called delayed-type hypersensitivity or DTH; asthma, chronic obstructive pulmonary disease (COPD), inflammatory bowel disease (IBD), Crohn's diseases, ulcerative colitis, multiple sclerosis, septic shock, HIV-infection, transplantation, graft-versus-host disease (GVHD).

Another object of the present invention is, therefore, the method for treating or preventing any of the above mentioned diseases by administering an effective amount RANTES, MIP-1alpha, MIP-1beta as well as their muteins having at least 90% homology with the corresponding wild-type (WT) together with a pharmaceutically acceptable excipient.

Another embodiment of the present invention is the method for treating MS and/or other demyelinating diseases by orally administering an effective amount of CC chemokines comprising at least two mutations in the cationic site of the so-called 40's loop together with a pharmaceutically acceptable excipient. More preferably these mutated CC chemokines comprise an amino acid sequence selected from the group consisting of SEQ ID 1, 2, 4, 5 and 6.

The "cationic site of the so-called 40's loop" is clearly shown for some CC chemokines in PCT/EP 01/11428 (see in particular Figure 1).

Data concerning the pharmacokinetics as well as the biological effects of such CC chemokines oral administration were not available prior to the present invention, which is based on a study designed so as to compare the pharmacokinetics as well as biological effect of such CC chemokines following parenteral and oral administrations.

The results show that the oral administration of such CC chemokines displays comparable biological effects with the parenteral administration.

This is particularly surprising because this is a first time that a peptide or a protein shows such a level of oral efficacy in animal model for MS.

The chemokines of the present invention can be administered to a patient in need, alone, or in pharmaceutical compositions where one or more of the chemokines are mixed with suitable carriers or pharmaceutically acceptable excipient(s) at doses to treat, ameliorate or prevent the disease.

The pharmaceutical composition may comprise other active ingredients in addition to the chemokines or the treatment with the chemokines may be combined with the treatment with other active ingredients, which are able to treat, ameliorate or prevent the same disease.

A "therapeutically effective" dose further refers to that amount of the compound sufficient to result in amelioration of symptoms. Techniques for formulation and administration of the compounds of the instant application may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, Pa., latest edition.

The pharmaceutical compositions of the present invention may be manufactured in a manner that is itself known, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

Pharmaceutical compositions for use in accordance with the present invention thus may be formulated in conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active molecules into preparations which can be used pharmaceutically.

For example, for oral administration, the active ingredient can be formulated readily by combining the active compounds with pharmaceutically acceptable carriers well known in the art. Such carriers enable the compounds of the invention to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a patient to be treated. Pharmaceutical preparations for oral use can be obtained with a solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical preparations, which can be used orally, include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. All formulations for oral administration should be in dosages suitable for such administration.

Pharmaceutical compositions suitable for use in the present invention include compositions wherein the active ingredients are contained in an effective amount to achieve its intended purpose. More specifically, a therapeutically effective amount means an amount effective to prevent development of or to alleviate the existing symptoms of the subject being treated. Determination of the effective amounts is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein. The exact formulation and dosage can be chosen by the individual physician in view of the patient's condition. See, e.g., Fingl et al., 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1 p.1.

Preferably, the dosage of the chemokines of the present invention as defined above is between about 10 μg to about 100 mg a day, more preferably from 0.05 to 10 mg per day. Moreover, the age, sex and physical condition of the patient, as well as other concurrent treatments being administered also have a bearing on the effective dosage of the chemokines for treatment. Consequently, adjustment and refinement of the dosages used and administration schedules must be determined based on these factors, and may need to be determined experimentally. Such determinations, however, require no more than routine experimentation.

It will be appreciated that unit content of active ingredient(s) contained in an individual dose of each dosage form need not in itself constitute an effective amount, since the necessary effective amount can be reached by administration of a plurality of dosage units (such as capsules or tablets or combinations thereof). Administration of an effective dosage may be in a single dose form or in multiple dosage forms and it may be provided with an enteric coating and/or a sustained release mechanism, such as a degradable matrix or a reservoir.

The compositions may, if desired, be presented in a pack or dispenser device which may contain one or more unit dosage forms containing the active ingredient. The pack may, for example, comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration.

Compositions comprising a compound of the invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition. Suitable conditions indicated on the label may include treatment of disorders or diseases in which the administration of a compound of the present invention is desired to ameliorate either the disease or disorder or symptoms related to such disease or disorder.

"Oral" administration includes oral, enteral or intragastric administration. In addition, synergists can be conjoined in the treatment to enhance the effectiveness of the above.

WT chemokines are marketed for research purposes and can be bought or synthesized in house without any particular difficulty. The muteins of RANTES, MIP-1alpha and MIP-1beta mentioned in the present application and the Examples can be prepared by any known methods. However, for the preparation of the RANTES mutants reference is made to PCT/EP 01/11428 and to EP 01000761.5 (both applications in the Examples' sections report complete and specific methods for the recombinant preparation of all the muteins cited herein) and for the preparation of the muteins of MIP-1alpha and MIP-1beta reference is made respectively to Koopmann W and Krangel MS., J Biol Chem. 1997, 272(15):10103-9) and Laurence JS, Biochemistry 2001, 40: 4990-4999).

The following are examples, which are intended to illustrate the present invention without limiting its scope. The examples will make references to some Figures as described below.

DESCRIPTION OF THE FIGURES

Figure 1 shows the detection of RANTES(R44AK45AR47A) vs. hRANTES in serum following i.p. (Figure 1a) or i.v. administration (Figure 1b).

Figure 2 shows the dose response curve of RANTES(R44AK45AR47A) i.p. blocking WT-RANTES-induced peritoneal cell recruitment.

Figure 3 reports the dose response curve of RANTES(R44AK45AR47A) p.o. blocking WT RANTES induced peritoneal cell recruitment.

Figure 4 shows the time course of RANTES(R44AK45AR47A) p.o. blocking WT RANTES induced peritoneal cell recruitment.

Figure 5 reports the time course of RANTES(R44AK45AR47A) p.o. vs i.p. blocking WT RANTES induced peritoneal cell recruitment.

Figure 6 shows that MIP-1beta(K45AR46AK48A) administered p.o. blocks WT MIP-1beta- induced peritoneal cell recruitment.

Figure 7 shows the results obtained after i.p and p.o. administration of RANTES(R44AK45AR47A) in the EAE model in mice.

EXAMPLES

Methods

Pharmacokinetic studies

Female Balb/c mice aged 8-12 weeks were dosed with 5 mg/kg of WT or mutant chemokine by intravenous (i.v.), intraperitoneal (i.p.) or oral (p.o.) (oral gavage) routes. Blood was sampled at various time points (n=3 mice per group), serum was collected and the PK profile of RANTES or RANTES(R44AK45AR47A) was obtained by ELISA, using a polyclonal anti human RANTES antibody pair (Pharmingen 20581D/20582D), which was set up in house to detect hRANTES or RANTES(R44AK45AR47A) and not endogenous mRANTES.

Peritoneal cell chemotaxis

Female Balb/c mice aged 8-12 weeks were pre-dosed – 4 h p.o. or – 30 min i.p. with 200 µl vehicle control (NaCl), WT or mutant chemokine. At t = 0, mice were dosed i.p. with 200 µl vehicle control (NaCl), WT or mutant chemokin e. Mice were sacrificed 18 h later, a peritoneal lavage performed and total cells collected counted using a haemocytometer.

Statistical analysis

Total cell counts from peritoneal lavage are expressed as individual counts with the group mean. Statistical significance was calculated using a one-way ANOVA with Bonferroni post-test by GraphPad Prism software (version 3.0) so that $p>0.05$ did not represent a significant difference, $p<0.05$ *, $p<0.05$ ** and $p<0.01$ ***.

Experimental Autoimmune Encephalomyelitis (EAE)

Immunization procedure

8-week old C57 BL/6NCrlBR female mice weighing 18-22 grams were immunized (day=0) by injecting s.c. in the back of the neck 0.1 ml of an emulsion containing 200µg myelin oligodendrocyte glycoprotein 33-35 (MOG_{35-55}) peptide (Neosystem, Strasbourg, France) in Complete Freund's Adjuvant (CFA) (with *Mycobacterium butyricum*, Difco, Detroit, U.S.A.) containing 0.25 mg of *Mycobacterium tuberculosis*. Before the s.c injection, they received a 200 µl i.v. injection of 300 ng pertussis toxin (List Biological Lab., Campbell, CA, U.S.A.) dissolved in phosphate-buffered saline (PBS) in the tail vein. On day 2 the animals were given a second i.p. injection of 300 ng of pertussis toxin in PBS.

This procedure results, starting approximately from day 8-10, in the appearance of a progressive paralysis, arising from the tail and progressively ascending up to the forelimbs.

Study design

The study involved groups of 10 animals each. All the groups were immunized with MOG_{35-55} peptide in CFA and pertussis toxin, according with the immunization protocol.

Group 1: positive control group dosed with vehicle alone (PBS) by i.p. route.

Group 2: positive control group dosed with vehicle alone (PBS) by p.o. route.

Group 3: dosed with 10 µg/mouse i.p. of RANTES(R44AK45AR47A)

Group 4: dosed with 100 µg/mouse p.o. of RANTES(R44AK45AR47A)

Group 5 : dosed with 20,000 U/mouse s.c. of mouse recombinant interferon beta (m-IFN-β)

Vehicle

PBS was used to dilute RANTES(R44AK45AR47A) and mIFN-β to the appropriate concentration.

Administration route

RANTES(R44AK45AR47A) and m-IFN-β were administered daily respectively by p.o. or i.p. and s.c. route at the volume of administration of 200 µl/mouse. Groups 1, 2 were dosed respectively p.o. and i.p. with 200 µl/mouse of PBS.

Duration of treatment

The treatment started for each animal at experimental day 7 (approximately 3 days before the usual occurrence of the disease) and then continued for 21 consecutive days (sacrifice of animals at experimental day 28)

Clinical observations

Starting from day 5 the animals were individually examined for the presence of paralysis by means of a clinical score as follows:

0 = no sign of disease
0.5 = partial tail paralysis
1 = tail paralysis
1.5 = tail paralysis + partial unilateral hindlimb paralysis
2 = tail paralysis + hindlimb weakness or partial hindlimb paralysis
2.5 = tail paralysis + partial hindlimb paralysis (lowered pelvi)
3 = tail paralysis + complete hindlimb paralysis
3.5 = tail paralysis + complete hindlimb paralysis + incontinence
4 = tail paralysis + hindlimb paralysis + weakness or partial paralysis of forelimbs
5 = moribund or dead

Results

Detection of RANTES(R44AK45AR47A) vs. hRANTES in serum following i.p. or i.v. administration (figure 1)

Following i.p. administration, RANTES(R44AK45AR47A) was detected in serum after 5 min, with a peak at 30 min of 0.46 µg/ml, followed by a decline in detection by 6 h. In contrast hRANTES was only detected at very low levels throughout the time course (fig.1a). hRANTES (1 µg/ml) and RANTES(R44AK45AR47A) (1.5 µg/ml) were detected at peak levels immediately following i.v. administration (5 min) and the serum profile of both was similar throughout the time course (fig.1b).

Detection of RANTES(R44AK45AR47A) in serum following p.o. administration (see Table 1)

RANTES(R44AK45AR47A) was administered p.o at a dose of 100 µg/mouse. RANTES(R44AK45AR47A) was detected in the serum of the animals by ELISA at a peak level of 5.86 ng/ml serum 4 h after p.o. administration. nd= not detected

Table 1	RANTES(R44AK45AR47A) ng/ml serum		
t post p.o.	Mouse 1	Mouse 2	Mouse 3
15 min	0.58	Nd	Nd
30 min	nd	Nd	Nd
1 h	nd	Nd	2.34
2 h	3.81	1.11	0.01
4 h	3.17	5.18	9.24
8 h	1.06	1.35	4.9

Dose response curve of RANTES(R44AK45AR47A) i.p. blocking WT RANTES induced peritoneal cell recruitment (figure 2)

Mice were pre-dosed i.p. and the experiment conducted as previously described. hRANTES increased the yield of peritoneal cells by approximately 2-fold compared with baseline. In contrast, RANTES(R44AK45AR47A) failed to recruit cells. A dose dependent inhibition of hRANTES induced recruitment was observed by RANTES(R44AK45AR47A).

Dose response curve of RANTES(R44AK45AR47A) p.o. blocking WT RANTES induced peritoneal cell recruitment (figure 3)

Mice received various concentrations of RANTES(R44AK45AR47A) or 200 µl NaCl controls p.o. and the experiment was conducted as previously described. RANTES(R44AK45AR47A) blocked the hRANTES induced cell recruitment in a dose dependent manner.

Time course of RANTES(R44AK45AR47A) p.o. blocking WT RANTES induced peritoneal cell recruitment (figure 4)

Mice received 5 mg/kg RANTES(R44AK45AR47A) or NaCl vehicle control p.o. at various time points prior to an i.p. injection of hRANTES or NaCl vehicle control and the experiment was conducted as previously described. RANTES(R44AK45AR47A) was effective at inhibiting cell recruitment when administered up to 24 h before RANTES.

Time course of RANTES(R44AK45AR47A) p.o. vs i.p. blocking WT RANTES induced peritoneal cell recruitment (figure 5)

In another series of experiments we compared the effect of RANTES(R44AK45AR47A) i.p. at 10 µg at various time points prior to an i.p. injection of hRANTES or NaCl vehicle control and the experiment was conducted as previously described. We show that RANTES(R44AK45AR47A) is effective for longer following p.o. dosing (see results of Figure 4 in comparison with results of Figure 5).

MIP-1beta(K45AR46AK48A) administered p.o. blocks WT MIP-1beta-induced peritoneal cell recruitment (figure 6)

MIP-1beta(K45AR46AK48A) was administered p.o. at various doses and the experiment was conducted as previously described. MIP-1beta(K45AR46AK48A) effectively inhibited MIP-1beta-induced cell recruitment at doses as low as 0.015 mg/kg.

Oral vs. intraperitoneal efficacy of RANTES(R44AK45AR47A) in EAE model in mice (figure 7)

RANTES(R44AK45AR47A) showed a beneficial effect in the murine EAE model when administered orally. The protein, at 100 µg/mouse administered daily p.o. demonstrated a better efficacy than the reference treatment, recombinant m-IFN-beta. The mean of the maximum clinical score reached during the experiment was also decreased. These results show a clear beneficial effect of the oral administration of RANTES(R44AK45AR47A), which reduces clinical signs of chronic EAE in mice after immunization with MOG. Therefore, RANTES(R44AK45AR47A) can be administered orally for the treatment or prevention, in chronic demyelinating diseases such as MS.

Discussion

Three amino acid substitutions in the loop formed by amino acids 40-49 of RANTES (designated RANTES(R44AK45AR47A) and having the amino acid sequence of SEQ ID NO: 1) results in a protein which exhibits reduced GAG binding properties (≤80%) in an *in vitro* heparin binding assay. This mutant is active at recruiting cells in an *in vitro* chemotaxis model, but is fully inactive *in vivo*. It is widely believed that chemokines use GAGs, which are present throughout the body on the endothelial cell surface, as well as on the leukocyte surface, to enhance receptor presentation, and possibly to increase local concentrations of chemokine.

The fact that a GAG binding mutant of RANTES, which retains its receptor binding, is unable to recruit cells *in vivo* supports this hypothesis. We have shown that this mutant RANTES is also able to block WT mediated cell recruitment to the peritoneum.

Surprisingly, both RANTES and RANTES(R44AK45AR47A) are detected in the serum following oral administration. Following this result, RANTES(R44AK45AR47A) was tested for its ability to block RANTES mediated cell recruitment following oral administration. At doses 10 fold higher than that required for i.p. administration, RANTES(R44AK45AR47A) is effective at blocking RANTES mediated peritoneal cell recruitment.

RANTES mediates its effects through the chemokine receptors CCR1 and CCR5. As for most chemokine receptors CCR1 and CCR5 bind other chemokines in addition to RANTES

Finally, a GAG binding mutant of MIP-1beta was tested, and shown that, as for RANTES(R44AK45AR47A), it is unable to recruit cells following an i.p. injection and is also effective at blocking WT induced peritoneal cell recruitment both by the i.p. and as suggested by preliminary data (n=1) also by the p.o. route.

Therefore we have shown that some chemokines are orally available and that the blocking properties of the GAG binding mutants remain effective following oral administration.

Here below we report a summary of the findings that we have cited above:

Pre-treatment	Route	Chemoattractant (i.p.)	Result
RANTES(R44AK45AR47A)	i.p.	RANTES	Inhibition
RANTES(R44AK45AR47A)	i.p.	MIP-1beta	Inhibition
RANTES(R44AK45AR47A)	p.o.	RANTES	Inhibition
MIP-1beta(K45AR46AK48A)	i.p.	MIP-1beta	Inhibition
MIP-1beta(K45AR46AK48A)	p.o.	MIP-1beta	Inhibition

The following Table 2 will clarify the identity of the sequences reported in the Sequence Listing and throughout the text.

Table 2

SEQ ID NO:	Sequence description
1	RANTES(R44AK45AR47A)
2	RANTES(R44AK45AR47A)truncated(3-68)
3	Met-RANTES(R44AK45AR47A)
4	RANTES(G32NR44AK45AR47A)
5	MIP-1-alpha(R18A-R46A-R48A)
6	MIP-1-beta(K45A-R46A-K48A)
7	Met-RANTES(G32N)
8	RANTES(G32N)
9	RANTES(G32N)truncated(3-68)
10	WT-RANTES
11	RANTES(3-68)
12	WT-MIP-1-alpha
13	WT-MIP-1-beta

CLAIMS

1) Use of RANTES, MIP-1alpha, MIP-1beta as well as their muteins having at least 90% homology with the corresponding wild-type (WT) molecule to produce a pharmaceutical composition for treating or preventing autoimmune and inflammatory diseases as well as bacterial and viral infections by oral administration.

2) The use according to claim 1 wherein the muteins comprise at least two mutations with respect to the WT corresponding molecule in their GAG-binding domain.

3) The use according to any preceding claim wherein the muteins have from 95% to 99% of homology with the corresponding WT molecule.

4) The use according to any preceding claim wherein the muteins comprise an amino acid sequence selected from the group consisting of SEQ ID Nos: 1, 2, 3, 4, 5 and 6.

5) The use according to any preceding claims wherein the mutein has the amino acid sequence of SEQ ID NO: 1 and the autoimmune disease is multiple sclerosis.

ABSTRACT

The oral efficacy of some chemokines is shown. In particular RANTES, MIP -1alpha, MIP-1beta as well as their muteins having at least 90% homology with the corresponding wild-type (WT) molecule are effective when administered orally.

Figure 1a: i.p. administration

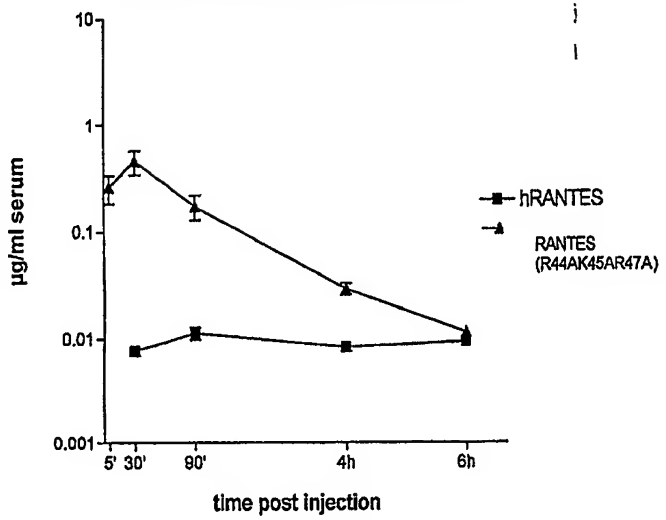


Figure 1b: i.v. administration

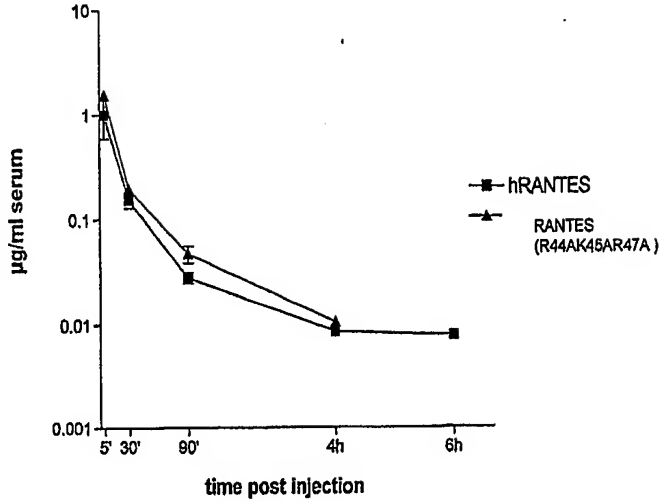
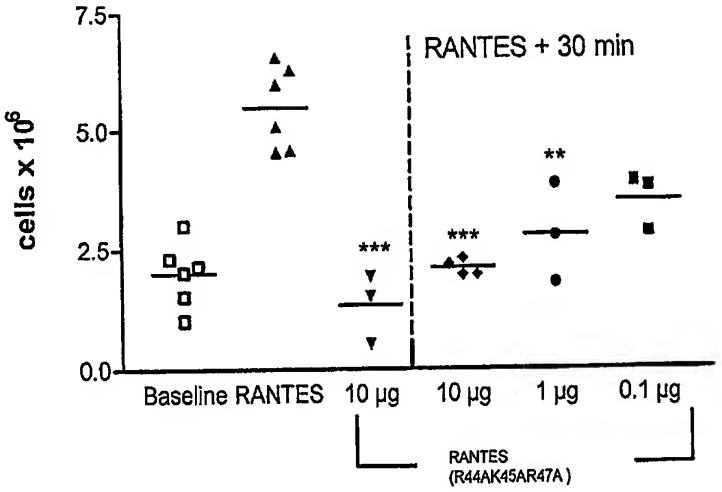


Figure 2


7.5
5.0
2.5
0.0
cells x 10^6
RANTES + 30 min
***
**
***
Baseline RANTES
10 µg
10 µg
1 µg
0.1 µg
RANTES
(R44AK45AR47A)

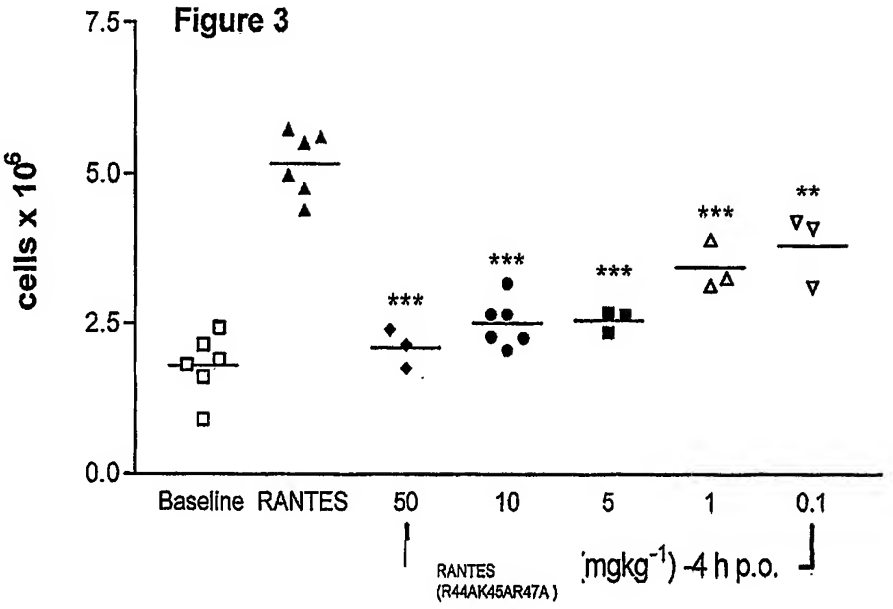


Figure 3
7.5
5.0
2.5
0.0
cells x 10^6
***
***
***
***
**
Baseline
RANTES
50
10
5
1
0.1
RANTES
(R44AK45AR47A)
(mgkg^{-1}) -4 h p.o.

Figure 4

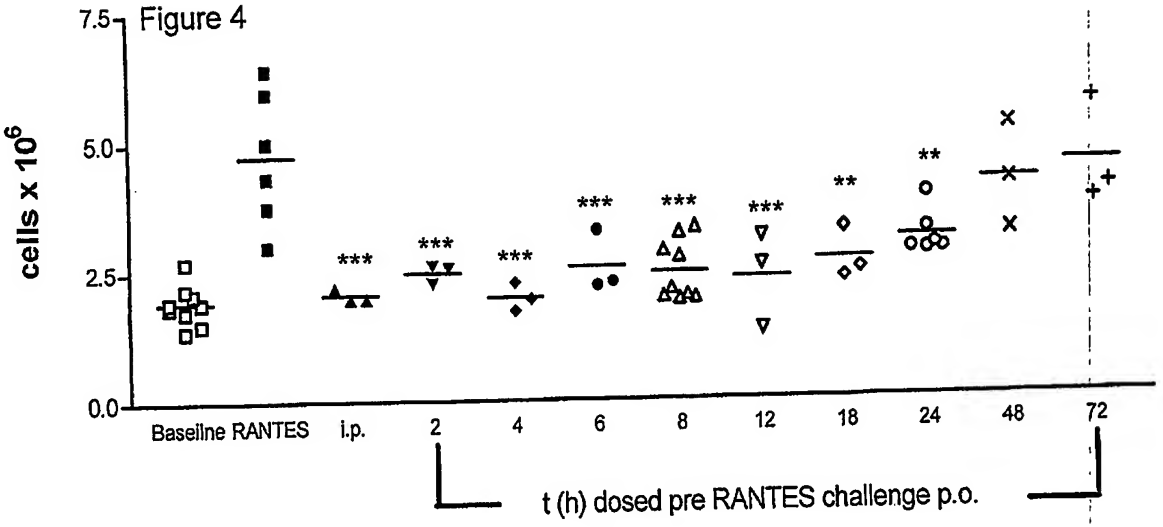

7.5
5.0
2.5
0.0
cells x 10^6
***
**
Baseline
RANTES
i.p.
2
4
6
8
12
18
24
48
72
t (h) dosed pre RANTES challenge p.o.

Figure 5

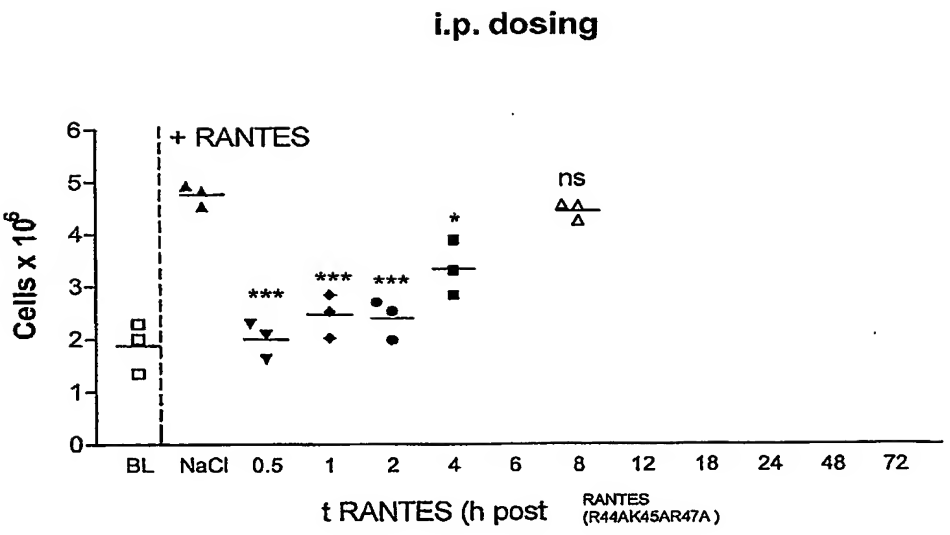

i.p. dosing
+ RANTES
ns
***
***
***
*
Cells x 10^6
0
1
2
3
4
5
6
BL
NaCl
0.5
1
2
4
6
8
12
18
24
48
72
t RANTES (h post
RANTES
(R44AK45AR47A)

Figure 6

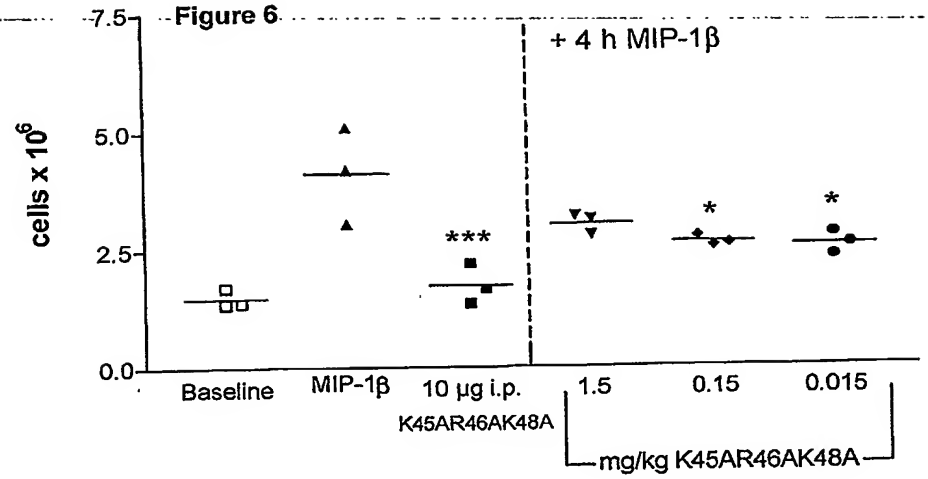
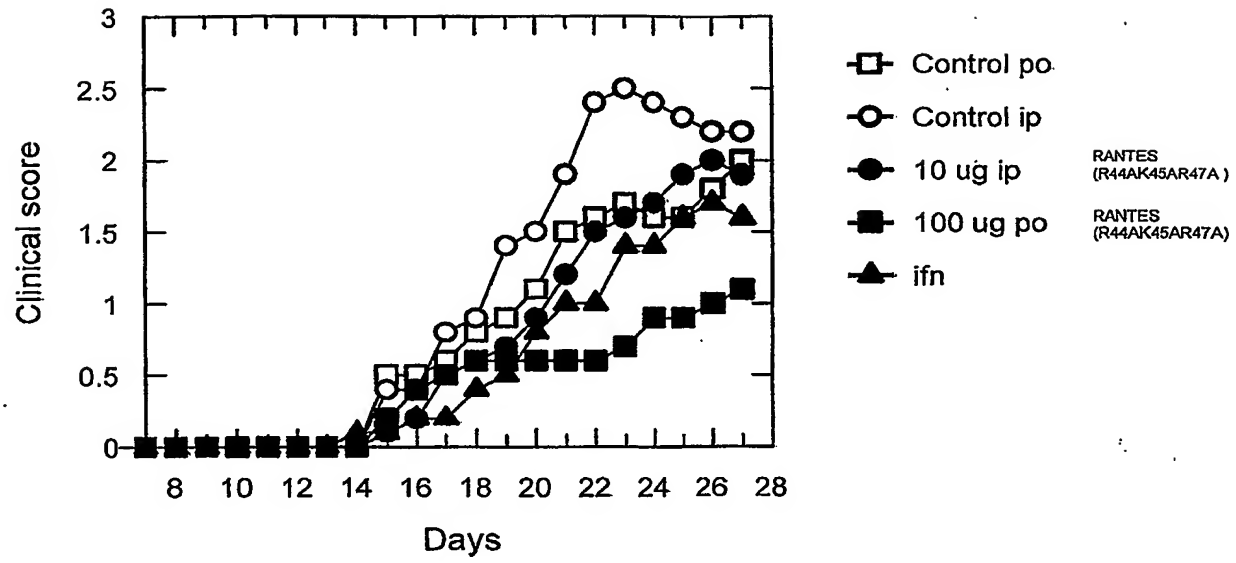

7.5
5.0
2.5
0.0
cells x 10^6
+ 4 h MIP-1β
***
*
*
Baseline
MIP-1β
10 µg i.p.
K45AR46AK48A
1.5
0.15
0.015
mg/kg K45AR46AK48A

Figure 7


Clinical score
3
2.5
2
1.5
1
0.5
0
8
10
12
14
16
18
20
22
24
26
28
Days
Control po
Control ip
10 ug ip
RANTES (R44AK45AR47A)
100 ug po
RANTES (R44AK45AR47A)
ifn

<110> APPLIED RESEARCH SYSTEMS ARS HOLDING N.V.

<120> PROTEINS HAVING IMPROVED ORAL BIOVAILABILITY

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<170> PatentIn version 3.1

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Cys Cys Phe Ala Tyr Ile Ala Arg Pro Leu Pro Arg Ala His Ile Lys
10                  15                  20                  25

Glu Tyr Phe Tyr Thr Ser Gly Lys Cys Ser Asn Pro Ala Val Val Phe
                30                  35                  40

Val Thr Ala Ala Asn Ala Gln Val Cys Ala Asn Pro Glu Lys Lys Trp
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Val Arg Glu Tyr Ile Asn Ser Leu Glu Met Ser
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20 25 30

Ser Asn Pro Ala Val Val Phe Val Thr Ala Ala Asn Ala Gln Val Cys
35 40 45

Ala Asn Pro Glu Lys Lys Trp Val Arg Glu Tyr Ile Asn Ser Leu Glu
50 55 60

Met Ser
65

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-5 -1 1 5

Pro Cys Cys Phe Ala Tyr Ile Ala Arg Pro Leu Pro Arg Ala His Ile
10 15 20 25

Lys Glu Tyr Phe Tyr Thr Ser Gly Lys Cys Ser Asn Pro Ala Val Val
30 35 40

Phe Val Thr Ala Ala Asn Ala Gln Val Cys Ala Asn Pro Glu Lys Lys
45 50 55

Trp Val Arg Glu Tyr Ile Asn Ser Leu Glu Met Ser
60 65

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-5 -1 1 5

Cys Cys Phe Ala Tyr Ile Ala Arg Pro Leu Pro Arg Ala His Ile Lys
10 15 20 25

Glu Tyr Phe Tyr Thr Ser Asn Lys Cys Ser Asn Pro Ala Val Val Phe
30 35 40

Val Thr Ala Ala Asn Ala Gln Val Cys Ala Asn Pro Glu Lys Lys Trp
45 50 55

Val Arg Glu Tyr Ile Asn Ser Leu Glu Met Ser
60 65

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Ser Gln Cys Ser Lys Pro Gly Val Ile Phe Leu Thr Lys Ala Ser Ala
35 40 45

Gln Val Cys Ala Asp Pro Ser Glu Glu Trp Val Gln Lys Tyr Val Ser
50 55 60

Asp Leu Glu Leu Ser Ala
65 70

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20 25 30

Cys Cys Phe Ala Tyr Ile Ala Arg Pro Leu Pro Arg Ala His Ile Lys
35 40 45

Glu Tyr Phe Tyr Thr Ser Gly Lys Cys Ser Asn Pro Ala Val Val Phe
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Val Arg Glu Tyr Ile Asn Ser Leu Glu Met Ser
85 90

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-5 -1 1 5

Pro Cys Cys Phe Ala Tyr Ile Ala Arg Pro Leu Pro Arg Ala His Ile
10 15 20 25

Lys Glu Tyr Phe Tyr Thr Ser Asn Lys Cys Ser Asn Pro Ala Val Val
30 35 40

Phe Val Thr Arg Lys Asn Arg Gln Val Cys Ala Asn Pro Glu Lys Lys
45 50 55

Trp Val Arg Glu Tyr Ile Asn Ser Leu Glu Met Ser
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Cys Cys Phe Ala Tyr Ile Ala Arg Pro Leu Pro Arg Ala His Ile Lys
10 15 20 25

Glu Tyr Phe Tyr Thr Ser Asn Lys Cys Ser Asn Pro Ala Val Val Phe
30 35 40

Val Thr Arg Lys Asn Arg Gln Val Cys Ala Asn Pro Glu Lys Lys Trp
45 50 55

Val Arg Glu Tyr Ile Asn Ser Leu Glu Met Ser
60 65

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        -5              -1  1               5

Phe Ala Tyr Ile Ala Arg Pro Leu Pro Arg Ala His Ile Lys Glu Tyr
10                  15                  20                  25

Phe Tyr Thr Ser Asn Lys Cys Ser Asn Pro Ala Val Val Phe Val Thr
                30                  35                  40

Arg Lys Asn Arg Gln Val Cys Ala Asn Pro Glu Lys Lys Trp Val Arg
            45                  50                  55

Glu Tyr Ile Asn Ser Leu Glu Met Ser
        60                  65
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<221> SIGNAL

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1               5                   10                  15

Leu Cys Ala Pro Ala Ser Ala Ser Pro Tyr Ser Ser Asp Thr Thr Pro
            20                  25                  30
```

Glu Tyr Phe Tyr Thr Ser Gly Lys Cys Ser Asn Pro Ala Val Val Phe
50 55 60

Val Thr Arg Lys Asn Arg Gln Val Cys Ala Asn Pro Glu Lys Lys Trp
65 70 75 80

Val Arg Glu Tyr Ile Asn Ser Leu Glu Met Ser
85 90

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Leu Cys Ala Pro Ala Ser Ala Tyr Ser Ser Asp Thr Thr Pro Cys Cys
-5 -1 1 5

Phe Ala Tyr Ile Ala Arg Pro Leu Pro Arg Ala His Ile Lys Glu Tyr
10 15 20 25

Phe Tyr Thr Ser Gly Lys Cys Ser Asn Pro Ala Val Val Phe Val Thr
30 35 40

Arg Lys Asn Arg Gln Val Cys Ala Asn Pro Glu Lys Lys Trp Val Arg
45 50 55

Glu Tyr Ile Asn Ser Leu Glu Met Ser
60 65

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<400> 12

[redacted] [redacted]

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1 5 10 15

Ser Arg Gln Ile Pro Gln Asn Phe Ile Ala Asp Tyr Phe Glu Thr Ser
20 25 30

Ser Gln Cys Ser Lys Pro Gly Val Ile Phe Leu Thr Lys Arg Ser Arg
35 40 45

Gln Val Cys Ala Asp Pro Ser Glu Glu Trp Val Gln Lys Tyr Val Ser
50 55 60

Asp Leu Glu Leu Ser Ala
65 70

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<211> 68

<212> PRT

<213> Escherichia coli

<400> 13

Ser Phe His Phe Ala Ala Asp Cys Cys Thr Ser Tyr Ile Ser Gln Ser
1 5 10 15

Ile Pro Cys Ser Leu Met Lys Ser Tyr Phe Glu Thr Ser Ser Glu Cys
20 25 30

Ser Lys Pro Gly Val Ile Phe Leu Thr Lys Lys Gly Arg Gln Val Cys
35 40 45

Ala Lys Pro Ser Gly Pro Gly Val Gln Asp Cys Met Lys Lys Leu Lys
50 55 60

Pro Tyr Ser Ile
65

10